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Cell-Specific Expression of the Diphtheria Toxin A-Chain Coding Sequence Under the Control of the Upstream Region of the Human Alpha-Fetoprotein Gene

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Background and Objectives: Development of the system to express a suicide gene selectively in tumor cells is essential for gene therapy. We constructed a plasmid containing the diphtheria toxin A (DTA) fragment linked to human alpha-fetoprotein (AFP) promoter and enhancer, and tested whether it can exert its cytocidal effect selectively on AFP-producing cells.
Methods: The chloramphenical acetyltransferase (CAT) reporter gene or DTA gene was linked to the 5′ upstream region of the AFP gene. The plasmids were transfected into AFP-producing or non-producing cells by the lipopolyamine-coated DNA method. Expression of CAT activity and effects on cell growth of transfected cells were assessed.
Results: When the AFP-producing cells HuH-7 or HepG2 were cotransfected with CAT reporter plasmid and pAF5.1DTA plasmid, the CAT activity was greatly suppressed. In contrast, cotransfection with pAF5.1DTA-R, the inversely inserted DTA gene, did not inhibit CAT activity. Furthermore, cell growth of HuH-7 cells transfected with pAF5.1DTA plasmid was significantly inhibited compared with HuH-7 cells transfected with DTA-R plasmid.
Conclusions: Our results indicate that selective killing of AFP-producing cells will be attained by introducing the DTA gene linked to the promoter and enhancer region of AFP.
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KEY WORDS: **alpha-fetoprotein; gene therapy; diphtheria toxin**

INTRODUCTION

The targeted expression of a suicide gene represents a powerful and novel approach to cancer therapy [1–5]. The diphtheria toxin (DT) gene has been cloned and sequenced and its mechanism of action already clarified [6–8]. DT is cleaved into two fragments, designated A and B chains, and is joined by a disulfide bridge [9]. The B subunit binds to the surface of most eukaryotic cells, where it is internalized by endocytosis and delivers the A chain (DT-A) into cells. Then, DT-A inactivates elongation factor 2, causing inhibition of protein synthesis [6,9–11]. Once inside the cell, DT-A is extremely toxic, and a single molecule is sufficient to kill a cell [12]. DT-A should be retained within the cells, and even if it were

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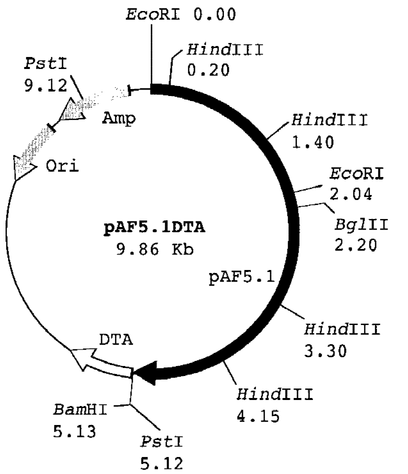

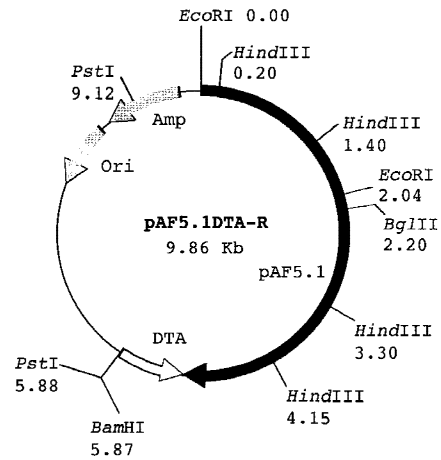

Fig. 1. Schematic representation of pAF5.1DTA and pAF5.1DTA-R plasmids. Shadowed region is the AFP 5′-flanking sequence. DTA-R is inversely inserted DTA.

released from dying cells, it would not enter other cells in the absence of the B chain [13]. Cell suicide by expression of the coding sequence for the diphtheria toxin A (DTA) polypeptide has been demonstrated in cell culture systems and transgenic animals [1,13–15], and this is applicable to cancer therapy. Selective expression may be achieved by linking the DT-A gene with tissue-specific transcriptional regulatory elements.

The alpha-fetoprotein (AFP) gene is a developmentally regulated gene whose activity decreases rapidly after birth and becomes hardly detectable in adult life. However, the AFP gene is often reactivated to a high level in hepatocellular carcinomas and teratomas [16–21]. The 5′-flanking region of the human AFP gene contains transcriptional control elements with characteristics of enhancers. In this study, we investigated whether the promoter and enhancer region of the human AFP gene is available for the expression of chloramphenicol acetyltransferase (CAT) and DT-A genes selectively in AFP-producing cells. The results were discussed from the viewpoint of future application of DT-A genes to the treatment of hepatoma cells.

MATERIALS AND METHODS

Plasmids and Plasmid Constructions

pAF5.1CAT [22] was supplied by T. Tamaoki (University of Calgary, Calgary, Canada). It contains the CAT sequence downstream of 5.1 kilobase pairs (kb) of the human AFP 5′-flanking sequence containing full enhancer and promoter regions. pLTR-DT, a DT-A plasmid under the control of the long terminal repeat (LTR) of bovine leukemia virus (BLV), was described previously [23]. pAF5.1DT-A plasmid was constructed by replacing the CAT segment at *Bam*HI site with the DT fragment obtained from pLTR-DT. The recombinant plasmid with correct insert orientation was termed pAF5.1DTA. Plasmid pAF5.1DTA-R is a control plasmid, with reverse insert orientation of DTA cassette (Fig. 1).

Cell Lines

HuH-7 [24] and HepG2 [25] are human AFP-producing hepatoma cell lines. NUE is also a human hepatoma cell line, but production of AFP is very low. MKN45 is a human CEA-positive gastric cancer cell line that does not produce AFP. The cells were maintained in culture flasks (Falcon 3024, Becton Dickinson, Oxnard, CA) in complete RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (Lot No. 40701, Common-Wealth Serum Lab., Victoria, Australia).

Transfections and Assay of CAT Activity

Transfection was carried out with lipopolyamine (TRANSFECTAM, Bio Sepra, Marlborough, MA) [26,27]. The lipopolyamine stock solution was obtained by 1:9 dilution of a 20 mM ethanolic solution with water. The DNA-lipid complex, freshly obtained by adding 250 μl of 5 μg of DNA solution to 250 μl of lipopolyamine solution containing 15 μl of stock solution and diluted in 4 ml of RPMI 1640 medium without fetal calf serum (FCS), was added to the 10^6 cells in 10 cm dish (Corning, Corning, NY). They were incubated at 37°C for 6 hr, followed by washing and addition of 10 ml complete medium containing 10% FCS. Cells were harvested 48 hr after cultivation in humidified 95% air/5% CO_2 incubator at 37°C. Cells were washed twice and transferred to an Eppendorf tube on ice, and extracts were prepared by freezing and thawing 3 times for CAT assay. CAT activity was measured according to the method of Gorman et al. [28]. After freeze/thaw cycles, 80 μl of the supernatant was added to 10 μl of Tris-HCl (pH 7.4) containing ^{14}C-labeled chloramphenicol. After 10 min at 37°C,

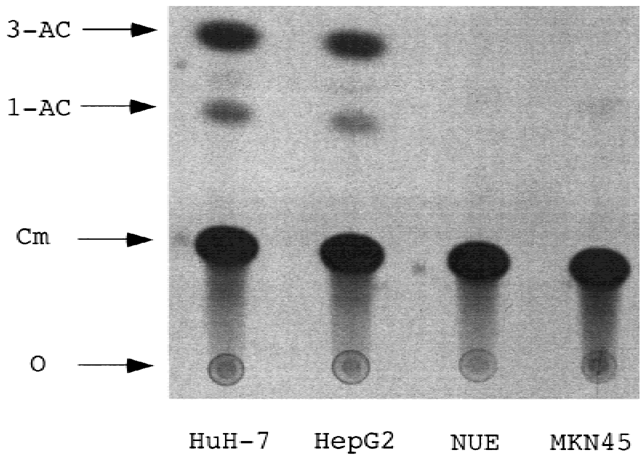

Fig. 2. Enhancer/promoter activity of AFP in various cell lines. Cells (1×10^6/dish) were seeded the day before transfection and were transfected with 5 μg of pAF5.1CAT mediated by lipopolyamine. Forty-eight hours after transfection, the cells were recovered and CAT activity was assessed. O, origin; Cm, chloramphenicol; 1-AC, 1-acetate chloramphenicol; 3-AC, 3-acetate chloramphenicol.

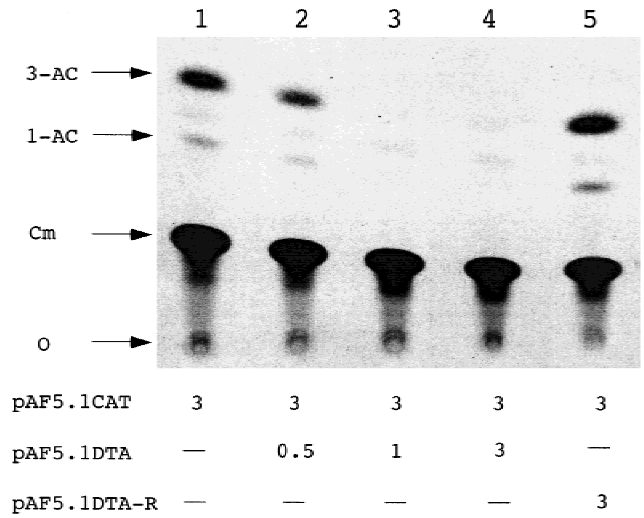

Fig. 3. Suppressive effect of pAF5.1DTA on the expression of CAT activity in HuH-7 cells. HuH-7 cells were cotransfected with 3 μg pAF5.1CAT **(lanes 1–5)** and various amounts of pAF5.1DTA **(lanes 2–4)** or pAF5.1DTA-R **(lane 5).** CAT activity was assessed 48 hr after transfection.

the reaction was initiated by adding 10 μl of 10 mM acetyl-CoA. After 1 hr at 37°C, chloramphenicol and its acetylated derivatives were extracted with ethyl acetate, separated by thin layer chromatography (TLC), and autoradiographed. The amount of proteins was determined using the Micro BCA protein assay kit (Pierce, Rockford, IL). In cotransfections, the DNA solutions contained 3 μg reporter plasmid (pAF5.1CAT) and the indicated amounts of the DT-A test plasmid (pAF5.1DTA) or 3 μg control plasmid (pAF5.1DTA-R).

Antitumor Effect of pAF5.1DTA In Vitro

HuH-7 cells (3×10^4) were seeded into each well of 6-well plates (Costar, Cambridge, MA). After overnight cultivation, the medium was removed and the cells were transfected with pAF5.1DTA or pAF5.1DTA-R plasmids. In this experiment, to reduce the cytotoxic effect of lipopolyamine, 1 μg of DNA and 1.5 μl of stock solution were mixed and the cells were incubated with DNA complex for 12 hr in 1 ml of medium without FCS. The number of recovered viable cells was assessed at days 1, 3, 5, and 7 of cultivation, and the results were expressed as the mean of 3 wells.

RESULTS

Enhancer and Promoter Activity of the AFP Gene

To assess the transcriptional activity of the AFP gene, various cell lines were transfected with pAF5.1CAT, which contains the coding sequence of bacterial CAT under the control of the AFP gene. As expected, HuH-7 cells, a high producer of AFP, and HepG2 cells, a moderate producer of AFP, showed CAT activity 48 hr after transfection (Fig. 2). In contrast, CAT activity was hardly detectable in the other 2 cell lines, NUE, a poor producer of AFP, and MKN45, a non-producer of AFP. While the efficiency of transfection varies from cells to cells, CAT activity was not detected in the latter 2 cell lines under the various conditions we tested (unpublished data). Furthermore, lacZ gene under the control of SR α promoter was expressed in all of the cells listed under the conditions described here (data not shown).

Suppression of CAT Activity in AFP-Producing Cells by Cotransfection With pAF5.1DTA Plasmid

We then tested whether pAF5.1DTA can work under the control of the upstream region of the AFP gene. HuH-7 cells were cotransfected with a fixed amount (3 μg/10^6 cells) of pAF5.1CAT and various amounts (0–3 μg/10^6 cells) of pAF5.1DTA. As shown in Figure 3, by increasing the amount of pAF5.1DTA, CAT activity in the cells drastically decreased. In contrast, addition of pAF5.1DTA-R did not affect transcription of the CAT gene. Similar results were obtained with HepG2 cells (Fig. 4). These results strongly indicate that the function of AFP-producing cells was suppressed when the DT-A gene was expressed under the control of AFP promoter and enhancer regions.

Inhibition of Cell Growth by DT-A

pAF5.1DTA or pAF5.1DTA-R plasmids were transfected into HuH-7 cells and their effects on cell growth were assessed by enumerating the viable cells at days 1, 3, 5, and 7 of cultivation. As shown in Figure 5, cell growth of pAF5.1DTA-transfected cells was significantly inhibited compared with that of HuH-7 cells transfected with pAF5.1DTA-R ($P < 0.05$).

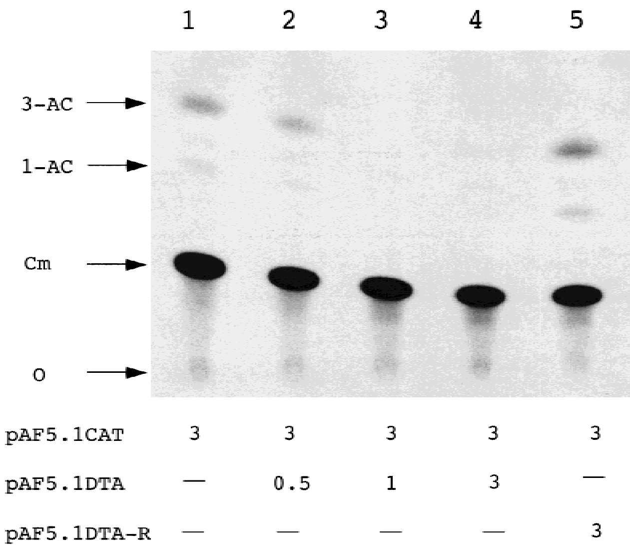

Fig. 4. Suppressive effect of pAF5.1DTA on the expression of CAT activity in HepG2 cells. HepG2 cells were cotransfected with 3 μg pAF5.1CAT **(lanes 1–5)** and various amounts of pAF5.1DTA **(lanes 2–4)** or pAF5.1DTA-R **(lane 5).**

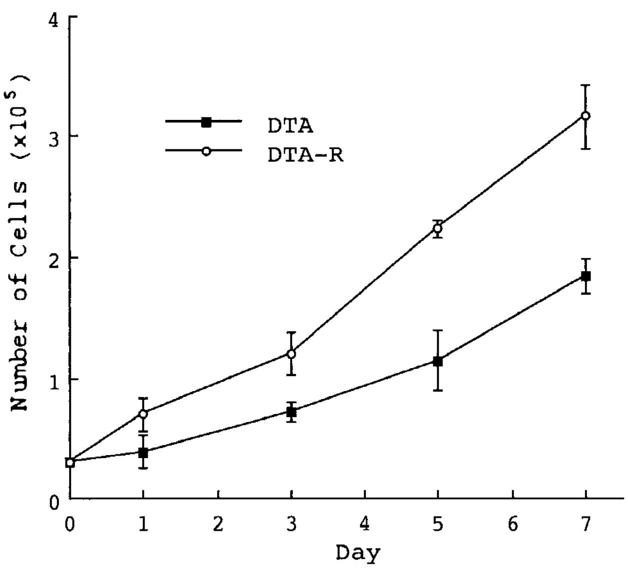

Fig. 5. Cell growth inhibition of HuH-7 cells transfected with pAF5.1DTA plasmid. HuH-7 cells (3×10^4) were seeded into each well of 6-well plates the day before transfection. The cells were transfected with pAF5.1DTA or pAF5.1DTA-R, and the number of viable cells was determined at days 1, 3, 5, and 7 of cultivation. The results were expressed as the mean cell number of 3 wells (significant at $P < 0.05$).

DISCUSSION

For gene therapy, there are two critical points for its efficacy, i.e., selective expression of the genes in the appropriate cells and development of an efficient vehicle to deliver an expression cassette of the genes to the target cells [29]. We demonstrated here that CAT activity could be selectively expressed in AFP-producing cells when the CAT gene was linked to the 5′-flanking region of the AFP gene. Furthermore, expression of CAT activity was suppressed by the DT-A gene with the AFP promoter/enhancer. These results strongly suggest that DT-A expression under the AFP promoter/enhancer is applicable to the selective treatment of AFP-positive hepatoma cells. Actually, we demonstrated here that inhibition of cell growth was evident in the AFP-producing HuH-7 cells transfected with the DT-A gene. In previous articles, we reported that DT-A under the control of LTR of BLV could inhibit the syncytium formation induced by BLV [23] and that the growth of BLV-infected cells was highly suppressed by successive treatment with pLTR-DT entrapped in positively charged liposomes [30]. These results also support that the DT-A gene linked to the tissue-specific transcriptional regulatory elements can exert its lethal effect. Use of the 5′-flanking region of the AFP gene for gene therapy was already begun by other investigators. Adenoviral vectors containing human AFP promoter/enhancer were used to express *Escherichia coli* cysteine deaminase or herpes simplex virus thymidine kinase selectively in AFP-producing hepatoma cells [31,32]. Furthermore, similar strategy is applicable to the cells that produce carcinoembryonic antigen [33] or prostate-specific antigen [34].

With regard to the vehicles of the genes, retrovirus and adenovirus are widely used [35,36]. They have many advantages but still some disadvantages are pointed out. The use of retrovirus vector is limited by the fact that target cells must proliferate in order to integrate the DNA into the genome, and a risk of insertional mutagenesis must be considered. Furthermore, this vector system is limited by low viral titer and low target cell transduction frequency. In contrast, adenovirus vectors are well suited for transfection, since they can be produced in high titers and efficiently transfer genes to replicating and non-replicating cells. However, adenovirus has some cytocidal effects, and in in vivo treatment, immune responses against adenovirus vectors will be quickly developed.

Liposomes are biodegradable and various molecules including monoclonal antibodies against tumor antigens could be conjugated for targeting therapy [37]. Moreover, by selecting an appropriate lipid for the construction of liposomes, the liposomes can exert cell fusion activity. Various cationic liposome preparations are now commercially available, but so far, these are used as plasmid-liposome complex, and in this experiment, too, such complexes were used for transfection. However, in the future, the plasmid DNA would be entrapped in the liposome for in vivo administration, and the surface of liposomes will be modified to endow the ability to deliver selectively to the target cells. Experiments are now under way to reach this goal.

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